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13. ABSTRACT *(Maximum 200 words)*

We examined the effects of the US Army Ranger Training Course (RTC), and increased (approximately 500 kcal/d) energy intake by Trainees on previously (1,2) reported suppressed T-lymphocyte functions and interleukin-6 (IL-6) levels. Blood samples were collected from Trainees at the beginning (Baseline) and during the Course; end of phases II, III and IV, and the middle of phase III. Blood cultures from the Trainees stimulated with phytohemagglutinin-M (PHA-M) showed reduced T-lymphocyte proliferation, release of soluble receptors for interleukin-2 (IL-2R), and production of IL-2 during the Course, with return to baseline values at the end of the RTC (phase IV). Effects of the RTC, likewise, resulted in reduced numbers of circulating blood T-lymphocyte subsets CD3 (total), CD4 (helper) and CD8 (suppressor), and levels of plasma and blood cell produced IL-6. Increased energy intake by Ranger Trainees of Class 11-92 (Ranger II Study) compared to 11-91 (Ranger I Study) resulted in less suppressed T-lymphocyte functions, but not the degree of reduced plasma IL-6. Thus, energy deficient experienced by the Ranger Trainees appears to have a suppressive affect on their T-lymphocyte immune functions. It is unclear if the suppressed plasma IL-6 levels is due to caloric deficit, high energy expenditure, or the combination.

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T-lymphocyte proliferation, Interleukin-2, Interleukin-2 receptors, Interleukin-6, Lymphocyte subsets, US Army Ranger Trainees

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Tina R. Kramer 4/13/93

PI - Signature Date

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INTRODUCTION

We concluded from a study conducted on volunteers of Ranger Class 11-91 that functions of the immune and inflammatory systems are suppressed in trainees during the US Army Ranger Training Course (1,2). The Ranger Trainees showed, compared to baseline, significantly ($p<0.05$) decreased proliferative responsiveness of T-lymphocytes in vitro to phytohemagglutinin-M (PHA-M) and of T and B-lymphocytes to pokeweed mitogen (PWM) upon completion of phase 1 (Benning phase), followed by additional and equivalent reductions upon completion of phases 2 (Mountain phase) and 3 (Swamp phase) of training (1,2). A partial, but significant ($p<0.05$), recovery in lymphocyte proliferation was observed at the end of phase 4 (Desert phase). We also reported (1,2), that compared to baseline, release of soluble interleukin-2 receptor (IL-2R) and production of interleukin-2 (IL-2) by whole blood cultures stimulated with PHA-M were reduced in the Trainees at the end of phases 1 and 2. Both interleukin functions were returned to baseline value at the end of phase 3. The concentration of supernatant IL-2 was increased above baseline level at the end of phase 4. We reported (1,2), in contrast to IL-2R and IL-2, proliferative responsiveness of T-lymphocytes to PHA-M from the Trainees was still suppressed at the end of phase 3, and only partially recovered to baseline at the end of phase 4. We also reported (1,2) that the Trainees showed significantly ($p<0.05$) increased plasma interleukin-6 (IL-6) at the end of phase 1, compared to baseline, followed by reduced levels at the end of phases 2 through 4, with the values at the end of phases 3 and 4 significantly lower than at baseline. It was hypothesized that prolonged energy-deficit was the likely cause of the reduction in immunocompetence.

Objective of the current study was to examine the effects of increased energy intake by US Army Ranger Trainees on suppressed T-lymphocyte functions and plasma IL-6 levels during the US Army Ranger Training Course.

METHODS

At each collection point, a 7 mL blood draw was collected, for immune studies, in sodium heparinized (143 USP units) VACUTAINER (Becton Dickinson VACUTAINER Systems, Rutherford, NJ; No. 6527, Lot No. 1Y061) tubes. The blood was held at ambient temperature (18-27^{o}C) for 20 h prior to preparation for in vitro cell culture, and staining and fixation of leukocyte subsets. Delayed processing was done to equalize the time for transport of the blood from the training sites to the immunology laboratory in Beltsville, Maryland. Plasma for IL-6 quantitation was removed from packed cells at 30-32 h postcollection. The plasma samples were held at -70^{o}C until analysis.

The blood was diluted 1:4 and 1:2 with RPMI-1640 tissue culture medium (Sigma Chemical Co., St. Louis, MO) in polystyrene tubes (FALCON^{R}, No. 2003, Becton Dickinson Labware) for preparation of lymphocyte proliferation and interleukin production cultures, respectively. The RPMI-1640 contained L-glutamine at 2.0 mmol/L and penicillin-streptomycin at 100,000 U/L and 0.1 mg/L, respectively; referred to heron as RPMI-1640. The in vitro cultures for lymphocyte proliferative responsiveness received in order: 100 uL of RPMI-1640 per well of round bottom 96-well tissue culture plates (Corning Glass Works, Corning, NY., Cat. No. 258550), 50 uL of RPMI-1640 alone (background) or with designated amounts of phytohemagglutinin-M (PHA-M; Sigma Chemical Co., St. Louis, MO., Cat. No. L-2646, Lot No. 49F-40221) or pokeweed mitogen (PWM; Sigma, Cat. No. L-9379, Lot No. 66F-9530). The cultures contained a final volume of 200 uL, with the final blood dilution at 1:16.

Proliferative activity in vitro was based on median DNA incorporation of tritiated thymidine (methyl-^{3}H; specific activity 6.7 uCi, 248 GBeq/mmol, DuPont, New England

Nuclear, Boston, MA) by cells in triplicate cultures without (background) and with stimulant. PHA-M was added to the cultures at 0.25, 0.5, 1, 2, 4, 8 and 16 ug per culture. PWM was added to the cultures at 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 ug per culture. Cell cultures stimulated with PHA-M were incubated for 72 h, and those with PWM for 144 h, at 37^{o}C in a 5% CO_2, 95% humidified air incubator. ^{3}H-thymidine (1.0 uCi; 37 KBeq) was added to each culture 24 h prior to termination. The cell cultures were harvested onto 12-well filtermats (Skatron Inc., Sterling, VA., Cat. No. 11731). The filter discs with 4.5 mL of scintillation fluid (READY-SAFE^{R}, Cat. No. 158735, Beckman, Columbia, MD) were counted in a beta scintillation counter (Beckman LS 3801). Proliferative activity of lymphocytes is expressed as mean DPM plus standard error.

Cells for interleukin production were cultured similar to those for proliferation, except that the cultures contained 50 uL of blood diluted at 1:2 with RPMI-1640, with a final dilution per culture at 1:8. The unstimulated cultures (background) received RPMI-1640 alone, and the stimulated cultures received 2.0 ug of PHA-M. The cultures were incubated for 48 h, at which time the supernatants from each set of six cultures were collected, pooled, and stored at -70^{o}C until assayed.

Interleukin-2 (IL-2) and soluble IL-2 receptors (IL-2R) were determined in supernatants from unstimulated (background) and PHA-M stimulated cultures by INTERLEUKIN-2 ELISA KITS (DuPont Co., Wilmington, DE; Cat. No. NEK-057, Lot No. C1049) and CELLFREE IL-2R Test Kits (T-CELL DIAGNOSTICS, Inc., Cambridge, MA; Cat. No. CK1024, Lot No. 5139), respectively. Interleukin-6 (IL-6) was determined in supernatants from unstimulated and PHA-M stimulated cultures, and plasma by Quantikine Immunoassay (R&D SYSTEMS, Minneapolis, MN; Cat No. D6050, Lot No. 9244181).

Lymphocyte subset markers for CD3, CD4, CD8 and CD19 were analyzed with a FACStar Facilitator (Becton Dickinson Immunocytometry Systems, Mountain View, San Jose, CA),

using the whole blood lysis method and dual color immunoflourescence assay. The results are presented as percent and absolute number of cells per uL of whole blood.

RESULTS

To determine the effects of the Ranger Training Course on proliferative responsiveness of T-lymphocytes from Trainees to suboptimal and optimal doses of PHA-M for maximum proliferation in vitro, whole blood cultures from volunteers of Class 11-92 (Ranger II) were stimulated with varying concentrations of PHA-M. When the cultures were stimulated with the optimal concentrations of PHA-M (2.0 through 16 ug/culture), the Trainees showed equivalent T-lymphocyte proliferation at baseline and at the end of the Course, phase 4 (Swamp phase), but significantly ($p < 0.05$) lower activity at the end of phases 2 (Desert phase) and 3 (Mountain phase), and at the middle of phase 3 (phase 2.5; Figure 1). When the cultures were stimulated with the suboptimal concentrations of PHA-M (0.5 and 1.0 ug/culture), they also showed significantly ($p < 0.05$) reduced T-lymphocyte proliferation at the end of phase 4 (Figure 2).

An objective of the present study was to determine the effect of increased energy intake on reported (1,2) suppressed T-lymphocyte proliferative responsiveness to PHA-M in Trainees during the Ranger Training Course. The mean percent suppressed T-lymphocyte proliferation from baseline for Trainees of the Ranger II study to 4.0 ug PHA-M per cell culture was less at the end of phases 2, 3 and 4 than for Trainees of Ranger I (Figure 3).

To reexamine the observation made in Ranger I Trainees of quicker return to baseline values of the early (IL-2R and IL-2) than late (DNA-synthesis) responses of T-lymphocyte activation/function during Ranger Training (1,2), soluble IL-2R release and IL-2 production were measured in supernatants of whole blood cultures stimulated with PHA-M from Ranger II Study Trainees (Figure 4). Compared to baseline, IL-2R and IL-2 levels were reduced in supernatants of PHA-M stimulated cell cultures from Ranger II Trainees at the end of phases

2 and 3, and at the middle of phase 3 (phase 2.5) of training (Figure 4; top and middle, respectively). In contrast to the Ranger I Study (1,2), IL-2R and IL-2 levels for Trainees of the Ranger II Study did not return to baseline value at the end of phase 3. The IL-2R, but not IL-2, levels returned to baseline value by the end of the Ranger Training Course, phase 4. The earlier observation made in the Ranger I Study (1,2) of quicker return to baseline value of the early (IL-2R and IL-2) than late (DNA-synthesis) responses of T-lymphocyte activation in Ranger Trainees is not consistent (Figure 4; bottom).

To determine the effects of the Ranger Training Course on blood leukocyte subsets of Ranger Trainees, blood samples from Ranger Trainees of the Ranger II Study were measured for leukocytes with subset markers CD3, CD4, CD8 and CD19. Compared to baseline, the percent CD3 positive T-lymphocytes and CD19 positive B-lymphocytes were decreased and increased, respectively, in the Trainees at the end of phases 2 and 4 of training (Figure 5). The percent CD4 and CD8 positive T-lymphocytes were increased and decreased, respectively, in the Trainees at phases 2.5, 3 and 4 of training, thus showing an increased ratio of CD4 to CD8 positive T-lymphocytes in the Trainees at these times (Figure 6). Compared to baseline, the absolute number of CD3, CD4 and CD8 positive T-lymphocytes per volume of blood were decreased in the Trainees at the end of training phases 2, 3 and 4 (Figure 7). While, at the middle of phase 3 (phase 2.5), following a short period of increased caloric intake, the absolute number of CD3 and CD8 positive T-lymphocytes per volume of blood were equivalent to baseline values, and the absolute number of CD4 positive T-lymphocytes was increased above baseline. The absolute number of CD19 positive B-lymphocytes per volume of blood were temporarily decreased in the Trainees at the end of phase 3 of training.

An additional objective of the present study was to determine the effect of increased energy intake on suppressed plasma IL-6 in Ranger Trainees. Trainees of Ranger II Study

showed a significant ($p < 0.05$) increase from baseline in plasma IL-6 at the end of phase 2 of the Ranger Training Course, followed by a return to baseline level by the middle of phase 3 (phase 2.5), with continued significant decreases at the end of phases 3 and 4 (Figure 8, top). Changes in plasma IL-6 for Trainees of the Ranger II Study were similar to those of Trainees in Ranger I Study (1,2). Thus, while increased energy intake by Trainees of Ranger II Study lessened the degree of suppressed T-lymphocyte proliferation in the Trainees to PHA-M, it did not reduce the degree of suppressed plasma IL-6 (Figure 9).

Similar to observations made in Ranger I Study, it appears that the change in plasma IL-6 during the Ranger Training Course for Trainees of the Ranger II Study were not due alone to the production of IL-6 by blood cells. Plasma IL-6 levels in Trainees of the Ranger II Study at phases 2.5 and 3 were equivalent to and decreased from baseline, respectively, while the IL-6 levels in supernatants from unstimulated whole blood cultures at the same times were increased and equivalent to baseline, respectively (Figure 8, bottom). Based on results from Ranger Studies I and II, we currently think that plasma IL-6 is a more accurate indicator of the effects of the Ranger Training Course on IL-6 status in Trainees than IL-6 levels in the supernatant of whole blood cultures.

CONCLUSIONS

The stresses of Ranger training results in reduced:

Proliferation of T-lymphocytes to PHA-M

Release of PHA-M induced soluble receptors for IL-2 (IL-2R)

Production of PHA-M induced IL-2

Absolute numbers of CD3, CD4 and CD8 positive T-lymphocytes

Levels of plasma and blood cell produced IL-6

Increased energy intake by Trainees of Ranger II Study:

Resulted in less suppressed T-lymphocyte proliferation to PHA-M

Did not lessen the degree of reduced plasma IL-6

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2. Kramer TR: Support between USAMRDC and USDA for cooperative research under the Ration Sustainment Testing Program. Army Project Order No. 91PP1807, US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND, Fort Detrick, Frederick, Maryland 21702-5012, December 31, 1992.

Figure 1

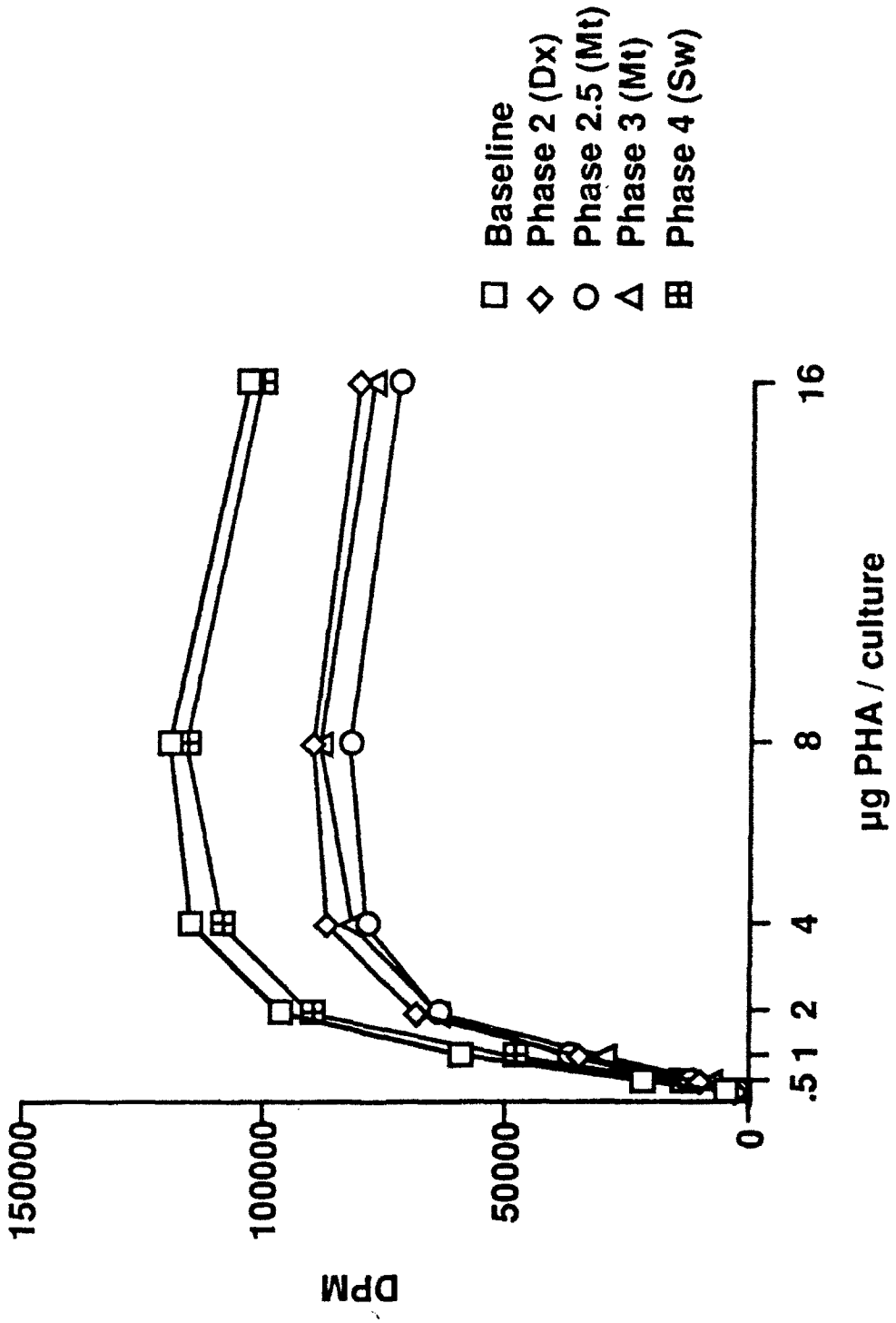
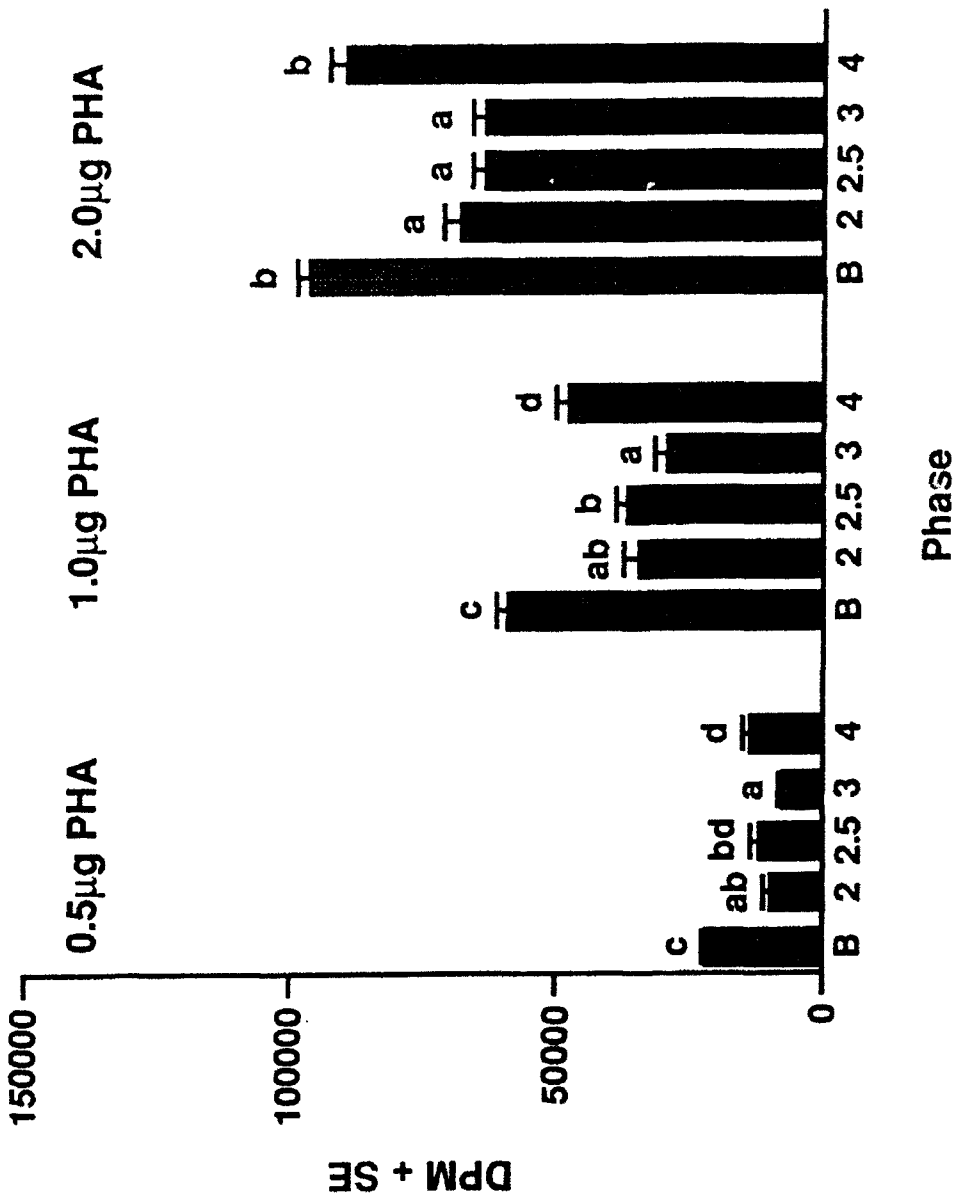

Baseline
Phase 2 (Dx)
Phase 2.5 (Mt)
Phase 3 (Mt)
Phase 4 (Sw)
DPM
150000
100000
50000
0
.5
1
2
4
8
16
µg PHA / culture

Figure 2


0.5µg PHA
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2.0µg PHA
DPM + SE
150000
100000
50000
0
B 2 2.5 3 4
Phase

Figure 3

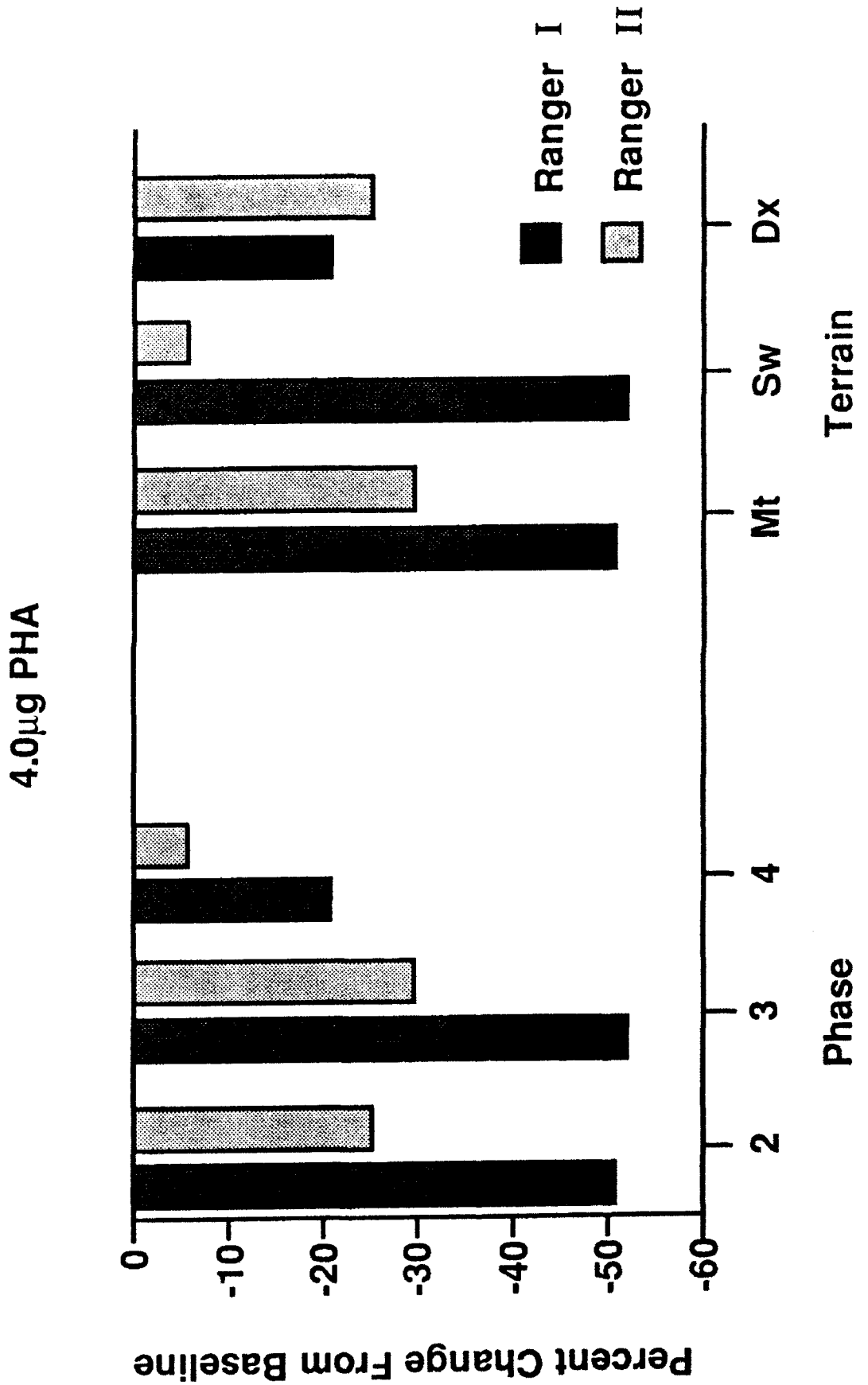

4.0µg PHA
Ranger I
Ranger II
Percent Change From Baseline
0
-10
-20
-30
-40
-50
-60
Phase
2
3
4
Terrain
Mt
Sw
Dx

Figure 4

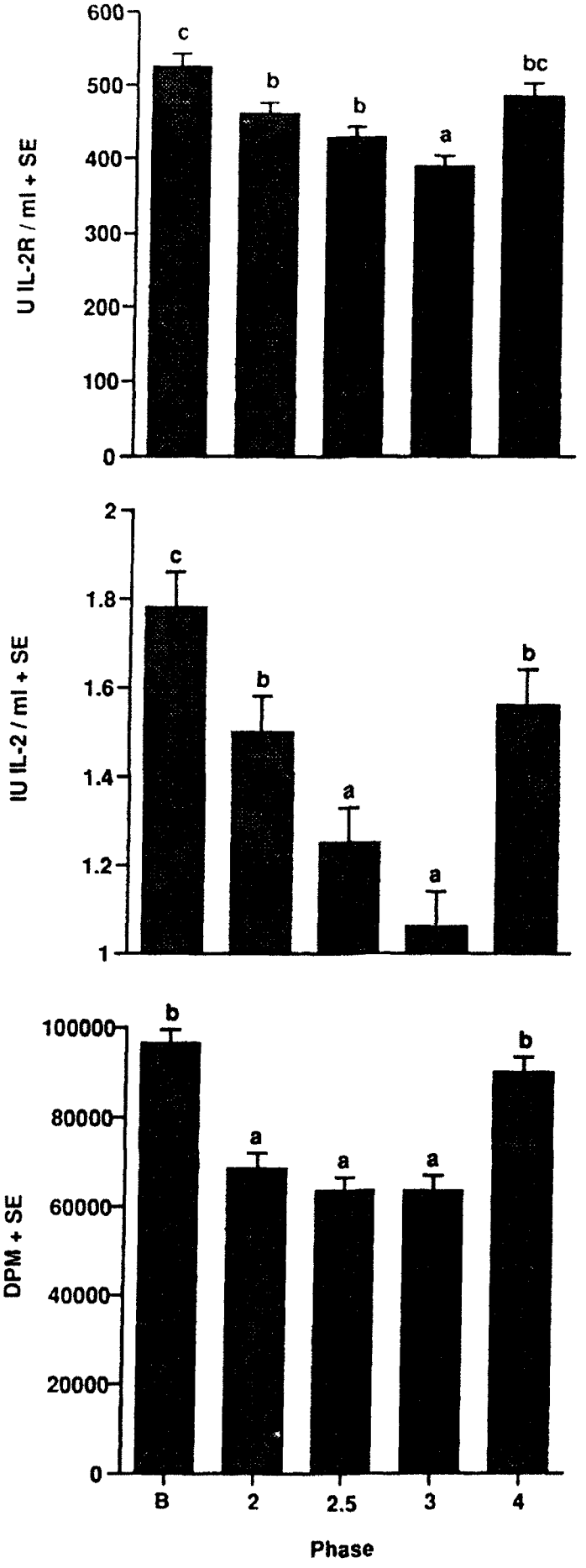

U IL-2R / ml + SE
600
500
400
300
200
100
0
c
b
b
a
bc
IU IL-2 / ml + SE
2
1.8
1.6
1.4
1.2
1
c
b
a
a
b
DPM + SE
100000
80000
60000
40000
20000
0
b
a
a
a
b
B
2
2.5
3
4
Phase

Figure 5

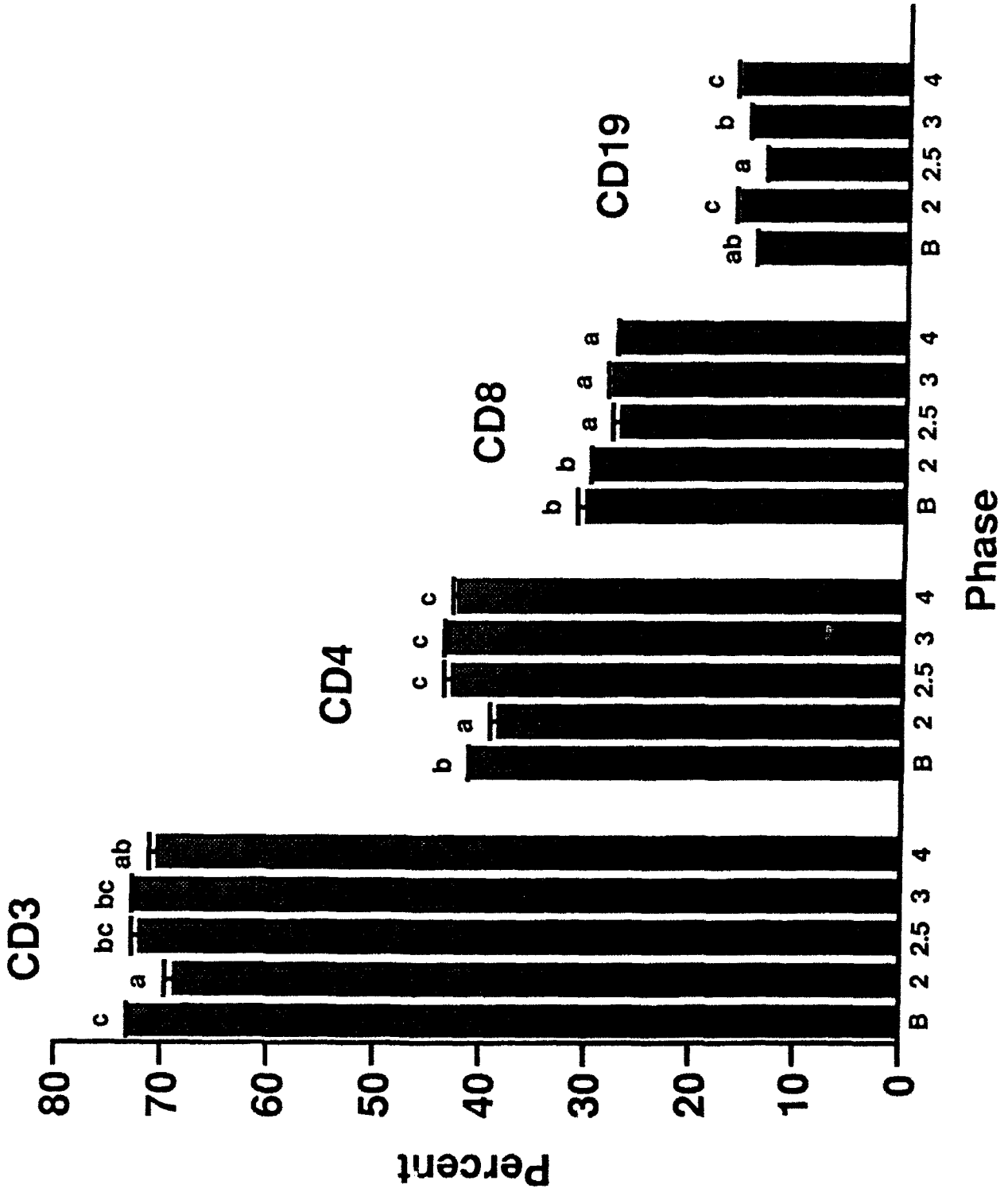

CD3
CD4
CD8
CD19
Percent
Phase
0
10
20
30
40
50
60
70
80
B 2 2.5 3 4
a
bc
bc
ab
c
b
c c c
b
a
b
b
a a a
ab
c
a
b
c

Figure 6

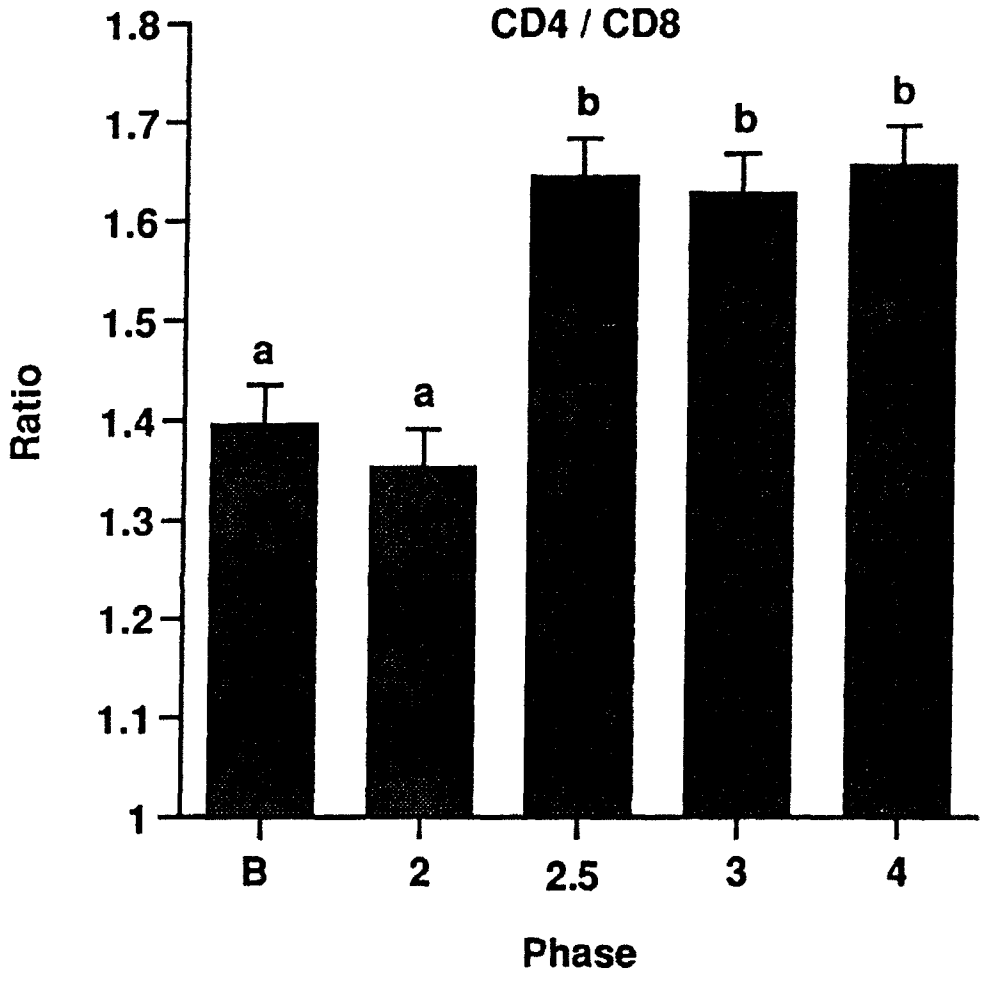


Figure 7

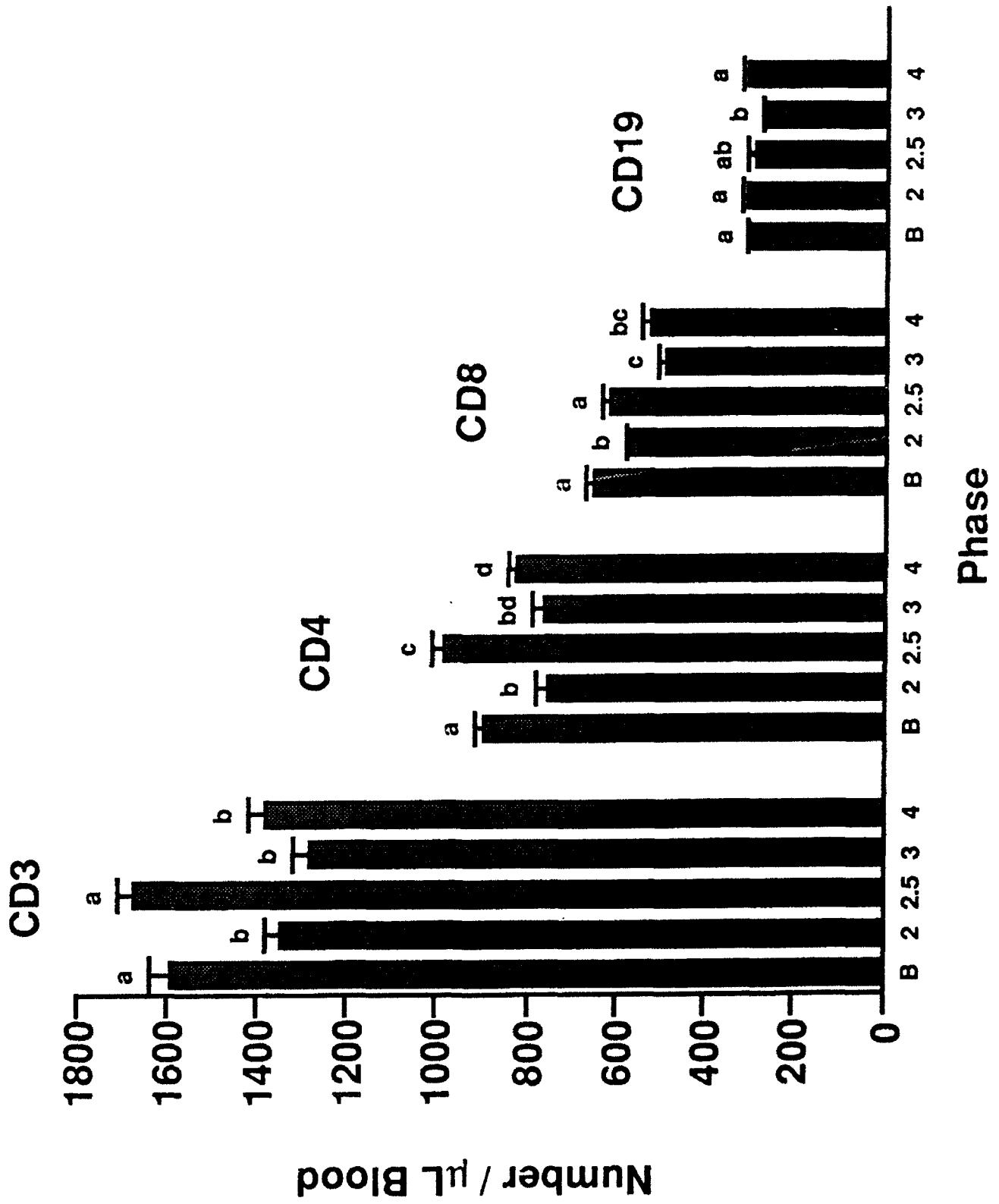

CD3
CD4
CD8
CD19
Number / µL Blood
Phase
1800
1600
1400
1200
1000
800
600
400
200
0
B 2 2.5 3 4

Figure 8

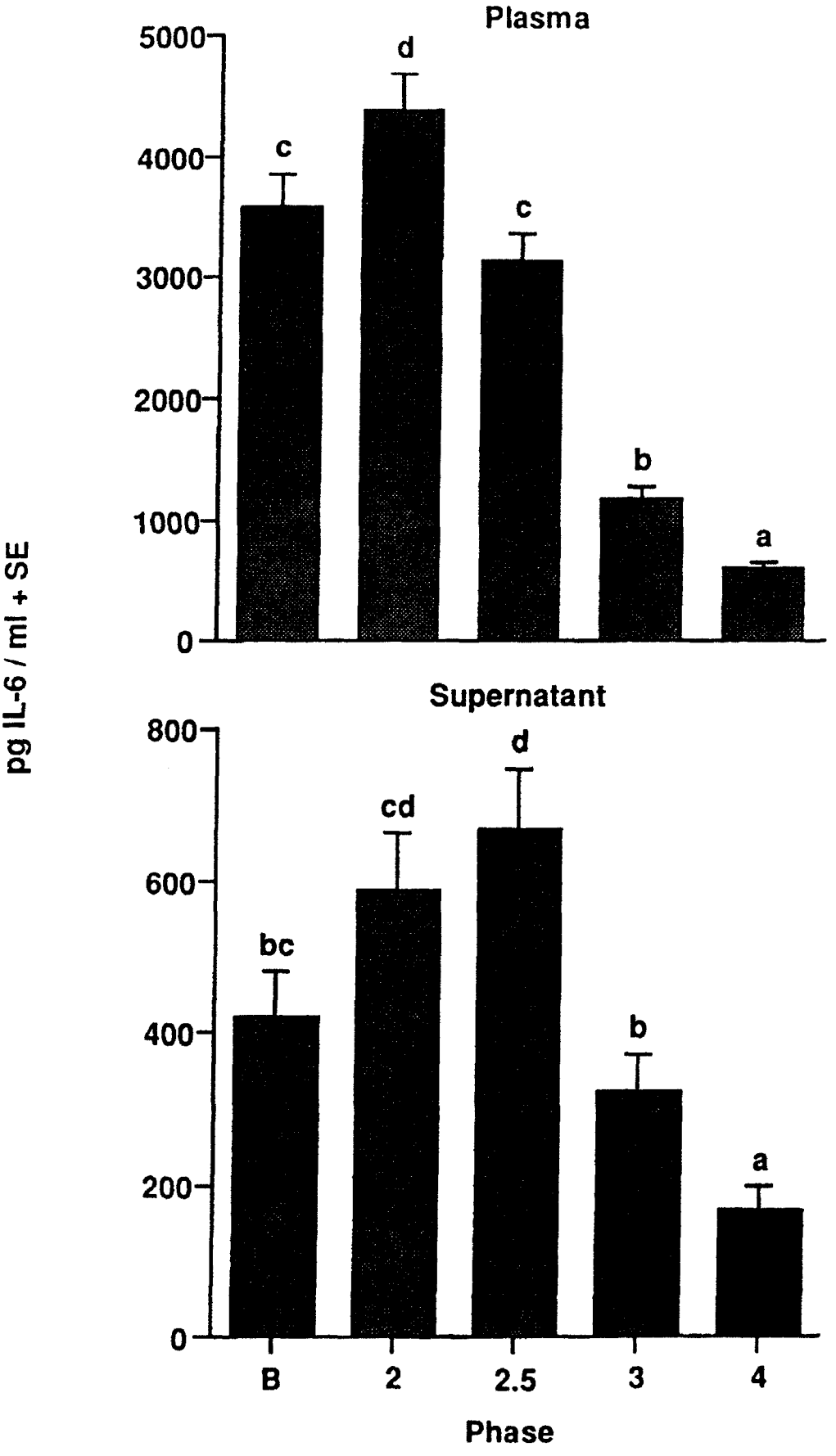
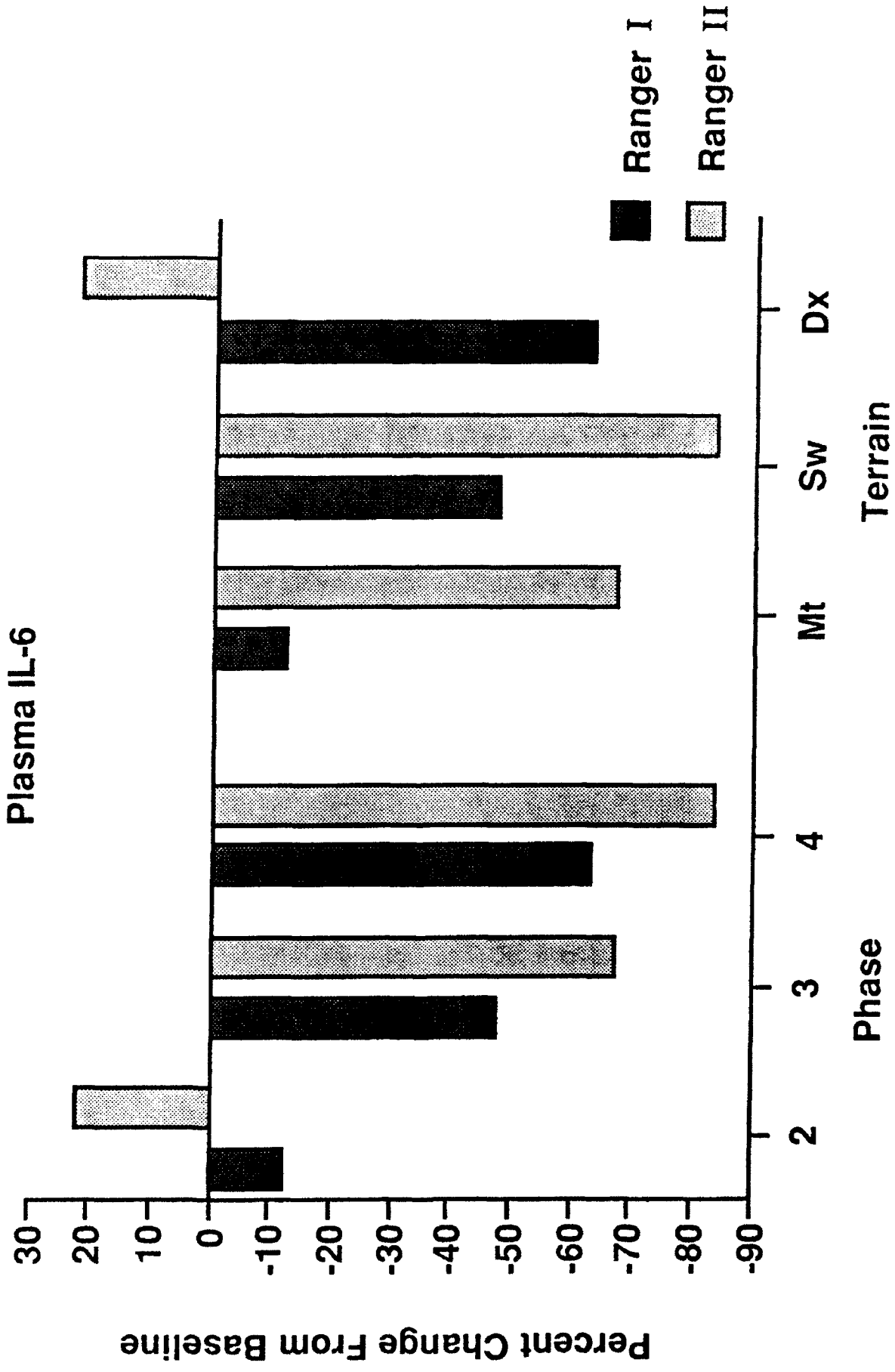

Plasma
5000
4000
3000
2000
1000
0
c
d
c
b
a
Supernatant
800
600
400
200
0
bc
cd
d
b
a
B
2
2.5
3
4
Phase
pg IL-6 / ml + SE

Figure 9


Plasma IL-6
Percent Change From Baseline
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
2
3
4
Mt
Sw
Dx
Phase
Terrain
Ranger I
Ranger II